

# Studies on Nucleotide and Receptor Regulation of $G_i$ Proteins: Effects of Pertussis Toxin

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In intact membranes as well as after reconstitution into phospholipid vesicles, pertussis toxin (PT)-mediated ADP-ribosylation of G proteins causes loss of receptor-mediated regulation of effectors and/or G protein-mediated regulation of receptor binding. Studies were carried out to test which of several discrete steps known to constitute the basal and receptor-stimulated regulatory cycles of  $G_i$  proteins are affected by PT. Experiments with the  $G_s$ -deficient  $G_i$ -regulated adenylyl cyclase of  $cyc^-$  S49 cell membranes indicated that PT blocks  $G_i$  activation by GTP without affecting GDP dissociation or GTP binding to a major extent. This suggested that the block lies in the transition of inactive GTP- $G_i$  to active GTP- $G_i$  (G to  $G^*$  transition). Experiments with purified  $G_i$  in solution and after incorporation into phospholipid vesicles showed that PT does not increase or decrease the intrinsic GTPase activity of  $G_i$ . Experiments in which  $G_i$  was incorporated into phospholipid vesicles with rhodopsin, a receptor that interacts with  $G_i$  to stimulate the rate of guanosine 5'-O-(3-thio)triphosphate binding and GTP hydrolysis, indicated that PT does not affect the basal GTPase activity of  $G_i$ , but blocks its activation by the photoreceptor. Taken together the results indicate that PT-mediated ADP ribosylation has two separate effects, one to block the interaction of receptor with  $G_i$  and another to impede the GTP-induced activation reaction from occurring, or that PT has only one effect, that of blocking interaction with receptors. In this latter case the present results add to a mounting series of data that are consistent with the hypothesis that unoccupied receptors are not inactive, but exhibit a basal agonist-independent activity responsible for the various effects of GTP observed on G protein-coupled effector functions in intact membranes. (*Molecular Endocrinology* 3: 1115-1124, 1989)

## INTRODUCTION

$G_i$  proteins are heterotrimers of subunit composition  $\alpha_i\beta\gamma$ , of which the  $\alpha$ -subunits migrate on sodium do-

decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels with apparent mol wt (Mr) of 40-41K and are ADP-ribosylated at a cysteine located four amino acids from their carboxyl-termini by pertussis toxin (PT). Molecular cloning has shown the existence of three types of  $G_i$  proteins,  $G_i$ -1,  $G_i$ -2, and  $G_i$ -3, characterized by having the same complement of  $\beta\gamma$  dimers and differing in their  $\alpha$ -subunits ( $\alpha_i$ -1,  $\alpha_i$ -2, and  $\alpha_i$ -3) (for review, see Refs. 1-3). The functions of  $G_i$  proteins are not totally clear at this time. The  $i$  subscript derives its origin from the fact that at the time the first PT-sensitive G protein (then thought to be only one) was purified (4-7), the only function known to be affected (blocked) by PT was hormonal inhibition of adenylyl cyclase (8-11). However,  $G_i$  preparations turned out to be heterogeneous, being generally a mixture of two, e.g.  $G_i$ -1 and  $G_i$ -2 in brain (12) and  $G_i$ -3 and  $G_i$ -2 in human erythrocytes (13) and white blood cells (14, 15), and possibly all three  $G_i$  proteins (16). Purified  $G_i$  proteins or their resolved guanosine 5'-O-(3-thio)triphosphate (GTP $\gamma$ S)-activated  $\alpha$ -subunits inhibit very little if any adenylyl cyclase activity in membranes (17, 18), and other functions have been defined which are mediated by PT-sensitive G proteins, including stimulation of  $K^+$  channels (19-22), which can be elicited with any one of the three  $G_i$  proteins (23), and stimulation of some phospholipases of the C (24) and  $A_2$  (25) types.

Receptor signal transduction by a G protein is thought to proceed through a complex regulatory cycle in which receptors associate with the trimeric G-GDP complex and promote the release of GDP, the binding of GTP, and the conformational change in the G protein that causes the receptor-G-GTP complex to undergo a two-step dissociation reaction, of which the first step is the separation of the  $\beta\gamma$  moiety and second step is the release of the receptor to give the activated  $\alpha$ -GTP ( $\alpha^*$ GTP). Receptors are then ready to initiate the activation of another G protein, while the activated G protein remains dissociated until  $\alpha$  hydrolyzes GTP to GDP, and the  $\alpha$ -GDP complex reassociates with  $\beta\gamma$  to give the receptor substrate G-GDP (discussed in Ref. 3). Just as the G protein undergoes changes during this cycle, so does the receptor, which adopts two conformations: one has high agonist affinity ( $R_H$ ) and is in-

duced by its interaction with the G protein, the other has low affinity for the agonist ( $R_L$ ) and represents free receptor not associated with G protein (26–31). In the absence of receptor stimulation by an agonist, this cycle proceeds much more slowly because of reduced rates of GDP release (32, 33) and a reduced rate of transition from G-GTP to  $G^*$ -GTP (34–36). Figure 1 depicts these regulatory cycles as applied to a PT-sensitive  $G_i$  protein.

Although the identity of the amino acid ADP-ribosylated by PT is known (37) and the effect of the modification is well recognized as one of impeding the receptor-mediated activation of the ADP-ribosylated G protein, be it inhibition of adenylyl cyclase (10, 38) or stimulation of  $K^+$  channels (19–22) or phospholipases (24, 25), there is little information about which of the steps of the receptor-mediated and GTPase-driven regulatory cycle of  $G_i$  proteins is affected by the toxin and which is not. Steps that have been shown to be affected include, in addition to the above-mentioned loss of receptor-mediated GTP-dependent regulation of effectors, the loss of ability of the  $G_i$  protein to confer high affinity agonist binding to receptors in intact membranes (39–42) as well as in phospholipid vesicles (30), and lack of  $G_i$  activation by GTP, as seen in *cyc*<sup>−</sup> membranes (11). These effects could be accounted for in several ways. PT may affect basic parameters of nucleotide- $G_i$  interaction, causing inhibition of GDP dissociation (step 1 in Fig. 1, A and B), inhibition of GTP binding (step 2 in Fig. 1, A and B), stimulation of GTP

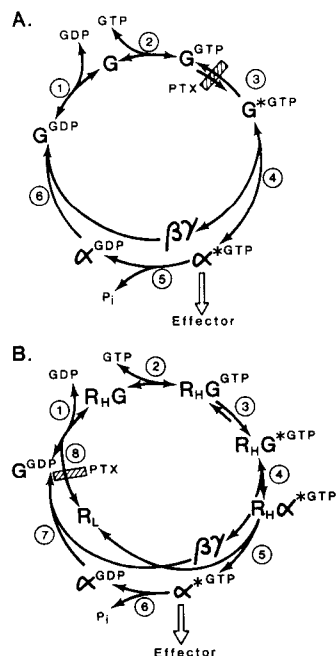
hydrolysis (step 5 in Fig. 1A and step 6 in Fig. 1B), and/or blocking GTP from inducing the conformational G to  $G^*$  change (step 3 in Fig. 1A). In addition to (or instead of) modifying the interaction of  $G_i$  with nucleotides, the ADP ribosylation could be a steric hindrance that impedes receptor from interacting with the  $G_i$  protein (step 8 in Fig. 1B).

The present studies were undertaken to gain further insight into steps of the above regulatory cycles that might be affected by PT, as seen in studies with intact membranes probing the  $G_i$ -mediated inhibition of adenylyl cyclase in S49 *cyc*<sup>−</sup> membranes which lack  $G_s$ , and in which the inhibition is due to the  $\alpha_i$ -GTP complex, and in studies in which the regulation of endogenous GTPase of a purified human erythrocyte  $G_i$  preparation (70%  $G_i$ -3 and 30%  $G_i$ -2) by photoactivated rhodopsin incorporated into phospholipid vesicles was evaluated. The first set of studies tested whether PT treatment interferes with the binding of GTP by reducing its affinity and/or with dissociation of GDP from the  $G_i$  protein. The second set of studies tested whether PT affects the intrinsic GTPase of the  $G_i$  protein as seen both in solution and after incorporation into phospholipid vesicles. These studies showed that in each of the two cycles PT affects only one step: step 3 (G to  $G^*$ ) in the absence of agonist and step 8 (association of  $R_L$  with G to give  $R_H G$  in the presence of agonist). The studies ruled out any significant effects on the other steps: GDP release, GTP binding, and GTPase activity.

## RESULTS

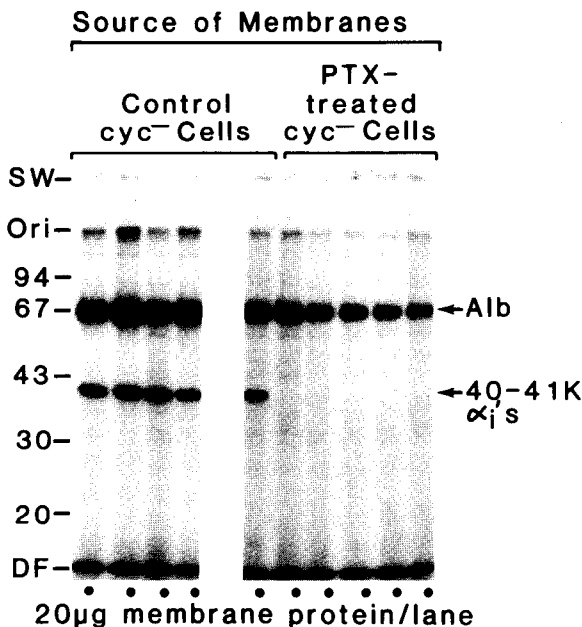
### Effect of PT on *Cyc*<sup>−</sup> Membrane $G_i$

In agreement with the results reported previously (43), *cyc*<sup>−</sup> S49 cells contain one or more PT-sensitive substrates which can be quantitatively ADP-ribosylated by treatment of cells overnight with 100 ng/ml of the toxin. Throughout these studies, membranes were prepared from both control and PT-treated cells on six occasions, five of which led to satisfactory modification of endogenous substrates, as assessed by the disappearance of G protein  $\alpha$ -subunits that could be ADP-ribosylated in the isolated membranes with [<sup>32</sup>P]nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and fresh PT (Fig. 2). In agreement also with results reported by us, GTP and guanylyl-5'-yl-imidodiphosphate [GMP-P(NH)P] inhibit *cyc*<sup>−</sup> adenylyl cyclase activity (43, 44), and treatment of *cyc*<sup>−</sup> S49 cells with PT blocked activation of  $G_i$  by GTP but not by GMP-P(NH)P (11). As shown in Fig. 4 for one of the sites of membrane preparations and confirmed with the four others, GMP-P(NH)P inhibited *cyc*<sup>−</sup> adenylyl cyclase activity in treated and control membranes to the same extent. However, it was less potent ( $IC_{50}$ , ~20 nM) in PT-treated cells than in control cells ( $IC_{50}$ , ~5 nM). Although the lack of an effect of GTP in membranes from PT-treated cells could have been due to a lack of GTP binding to  $G_i$ , this was not the case, as can be seen in Fig. 4. In this figure GTP is shown to



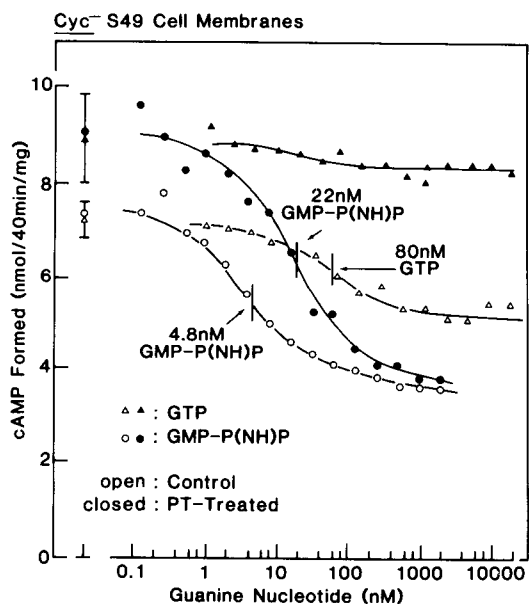
**Fig. 1.** Representation of the Regulatory Cycles of a  $G_i$  Protein as They Are Thought to Occur in the Absence (A) and Presence (B) of a Receptor

For discussion, see Ref. 32. ▢, Steps affected by PT (PTX). Steps 1, 2, and 5 in A and 6 in B were ruled out by the present studies.



**Fig. 2.** ADP-Ribosylation of Five Batches of Membranes from Each Control and PT-Treated  $Cyc^-$  S49 Cells with [ $^{32}P$ ]NAD $^+$  and 10  $\mu$ g/ml Activated PT

For details, see *Materials and Methods*.

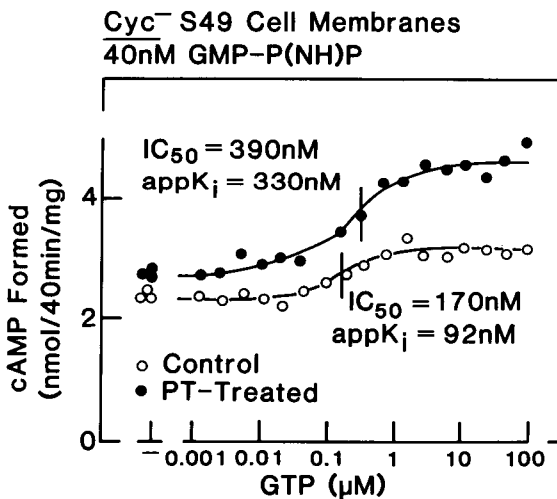


**Fig. 3.** Inhibitory Effect of Guanine Nucleotides on Control and PT-Treated  $Cyc^-$  S49 Cell Adenylyl Cyclase Activity

Membranes from control and PT-treated  $cyc^-$  cells were incubated for 40 min, as detailed in *Materials and Methods*, in the presence of the indicated guanine nucleotide concentrations, and the cAMP formed was measured. Note that nonhydrolyzable GTP analogs cause more inhibition than the hydrolyzable GTP in membranes from control cells, while in those from PT-treated cells only the nonhydrolyzable analog GMP-P(NH)P inhibits adenylyl cyclase activity. Values on the figure represent  $IC_{50}$  values.

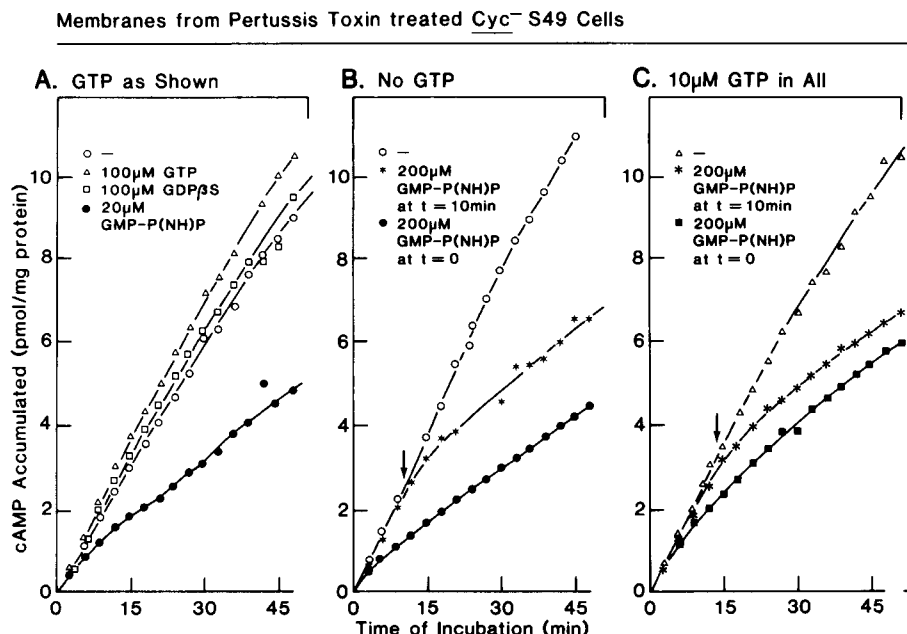
inhibit the GMP-P(NH)P-dependent inhibition of adenylyl cyclase activity in both PT-treated and control  $cyc^-$  membranes. As was the case for adenylyl cyclase inhibition by GMP-P(NH)P, both the  $IC_{50}$  as well as the apparent  $K_i$  for the action of GTP to block the GMP-P(NH)P effect were about 3-fold higher in PT-treated than control membranes.

Since these results suggest that GTP binding of  $G_i$  and GTP hydrolysis are not inhibited by PT treatment of  $cyc^-$  cells, we theorized that PT could alter the kinetics of the  $G_i$  regulatory cycle by inhibiting the GDP dissociation from the  $G_i$ -GDP complex. The accumulation of  $G_i$ -GDP complexes in PT-treated  $cyc^-$  membranes was assessed as described below, and the results obtained are presented in Fig. 5. Membranes from PT-treated  $cyc^-$  cells were incubated in the presence of guanine nucleotides, and the rate of cAMP formation measured. Figure 5A shows that incubation of the membranes with 100  $\mu$ M GTP had no effect on cAMP accumulation. However, incubation with 20  $\mu$ M GMP-P(NH)P resulted in a decrease in cAMP production, indicating that the nonhydrolyzable analog of GTP activates  $G_i$ , which, in turn, inhibits  $cyc^-$  membrane adenylyl cyclase activity. Activation of  $G_i$  can be detected after 10-min incubation in the absence of guanine nucleotides as measured by GMP-P(NH)P inhibition of cAMP formation (Fig. 5B). Figure 5C shows inhibition of cAMP formation by GMP-P(NH)P added after 10 min of incubation of membranes with 10  $\mu$ M GTP and indicates that preexposure of PT-treated  $G_i$  to GTP does not inhibit activation of  $G_i$  by GMP-P(NH)P. These re-



**Fig. 4.** GTP Can Block GMP-P(NH)P Inhibition of  $Cyc^-$  Membrane Adenylyl Cyclase Activity in Both Control and PT-Treated Cells

$Cyc^-$  S49 membranes from PT-treated and control cells were incubated for 40 min in the presence of 40 nM GMP-P(NH)P and the indicated concentrations of GTP, and the cAMP formed was assayed as indicated in *Materials and Methods*. Note that GTP blocks the inhibition by GMP-P(NH)P in both control and PT-treated  $cyc^-$  membranes. The apparent  $K_i$  values for the action of GTP were calculated according to the method of Cheng and Prusoff (72).



**Fig. 5.** GTP Does not Inhibit in a Persistent Manner GMP-P(NH)P-Mediated Activation of PT-Treated  $G_i$

Membranes from PT-treated *cyc*<sup>-</sup> S49 lymphoma cells were incubated for the indicated times, and the accumulation of cAMP was measured as described in *Materials and Methods*. The times of addition of GTP, GMP-P(NH)P, and GDPβS are indicated. Note that GTP is unable to cause the inhibition of *cyc*<sup>-</sup> adenyl cyclase activity, while GMP-P(NH)P does. Furthermore, addition of GMP-P(NH)P to the incubation mixture after 10-min incubation results in inhibition of cAMP formation regardless of the prior presence of GTP.

sults indicate that the inefficiency of GTP to activate  $G_i$  was not due to an arrest of the cycle in the  $G_i$ -GDP form.

### Studies with Purified $G_i$

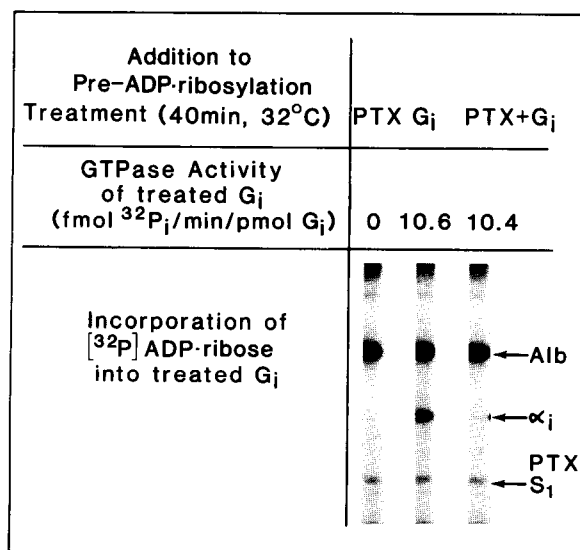
The results described above imply that PT does not block the GTPase reaction of one of its substrates,  $G_i$ . Since purified  $G_i$  proteins, in this case a mixture of 70%  $G_i$ -3 and 30%  $G_i$ -2 (Fig. 5 of Ref. 45), are also PT substrates (5) and exhibit basal GTPase activity in the absence of a functional receptor (46), we sought to confirm this conclusion by studying the effect of PT on the GTPase activity of purified  $G_i$  and on the dissociation of GDP from this protein mixture.

The efficacy of PT-mediated ADP-ribosylation of purified  $G_i$  was assessed by reincubating pretreated  $G_i$  with PT in the presence of [<sup>32</sup>P]NAD<sup>+</sup> in order to detect any further incorporation of [<sup>32</sup>P]ADP-ribose into protein. As shown in Fig. 6, ADP-ribosylated  $G_i$  incorporated less than 10% of the radiolabeled substrate incorporated by untreated  $G_i$ , indicating that under these conditions more than 90% of the protein was ADP-ribosylated. As illustrated in Fig. 6, the rates of inorganic <sup>32</sup>P (<sup>32</sup>P<sub>i</sub>) formation from GTP of untreated and ADP-ribosylated  $G_i$  were not significantly different, indicating that GTP hydrolysis by  $G_i$  is unaffected by PT treatment. Substitution of GDP for GTP in the ADP-ribosylation reaction, which does not alter the efficacy of the modification (47), did not change the results (data not

shown). These results indicate that the GTPase activity of  $G_i$  is not inhibited by preincubation with GDP and, therefore, suggest that PT does not prevent GDP dissociation from the  $G_i$ -GDP complex.

### Effect of PT on $G_i$ -Receptor Interaction

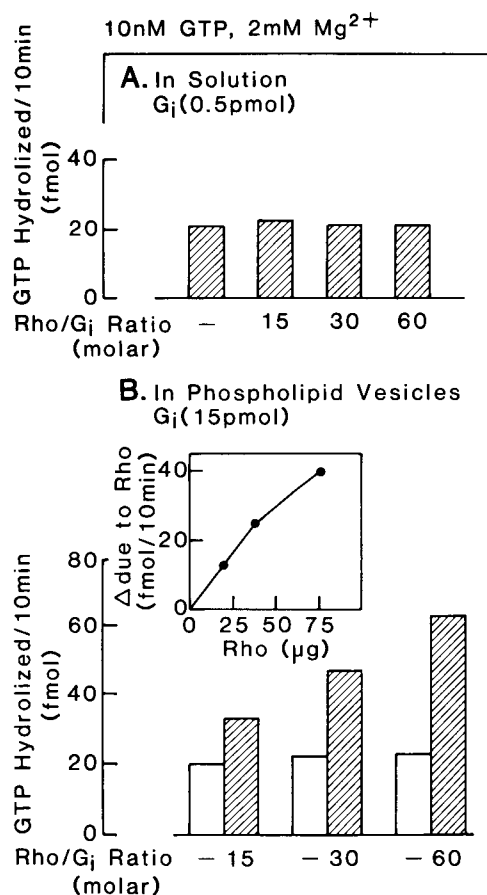
We next studied the interaction of PT-treated ADP-ribosylated  $G_i$  with a receptor. Previous studies had demonstrated that photoactivated rhodopsin can stimulate the GTPase activity of the  $G_i$  preparation used in this study (48). Rhodopsin was purified from bovine retina and used to study its interaction with ADP-ribosylated  $G_i$ . Figure 7 shows that the interaction between  $G_i$  and a receptor (rhodopsin in this case) can be measured only when both proteins are integrated into phospholipid bilayer, as previously reported for this and other G proteins (29, 48, 49). GTPase activity of  $G_i$  was measured in the absence or presence of different rhodopsin concentrations, before incorporation, *i.e.* in detergent solution (A), or after incorporation into phospholipid vesicles (B). The GTPase activity of  $G_i$  was stimulated by rhodopsin in proportion to the amount of photoreceptor used only when both proteins were co-reconstituted into the vesicles. When rhodopsin was incorporated into phospholipid vesicles with ADP-ribosylated  $G_i$ , no stimulation of GTPase by the photoreceptor could be detected at any of three rhodopsin/ $G_i$  ratios tested (Fig. 8). The lack of effect of rhodopsin was not due to altered incorporation of proteins into



**Fig. 6.** Lack of effect of PT-mediated ADP-Ribosylation of  $G_i$  on Its GTPase Activity

One microgram of  $G_i$  (10 pmol) was incubated for 45 min at 32 C with 2 mM  $NAD^+$ , 0.1 mM ATP, 1  $\mu$ M GTP, 0.01% BSA, 1 mM EDTA, and 12.5 mM Tris-HCl, pH 7.5, in the absence or presence of 2  $\mu$ g PT in a final volume of 50  $\mu$ l. As a control, PT was incubated under the same conditions without  $G_i$ . Aliquots (2  $\mu$ l) of each group were then diluted 20 times and subjected to a second ADP-ribosylation using  $[^{32}P]NAD^+$ , and in parallel, 10  $\mu$ l from each group were assayed for GTPase activity. The GTPase assays were performed in the presence of 100 nM GTP (carried over from the pre-ADP-ribosylation treatment step) under the conditions detailed in *Materials and Methods*. The second ADP-ribosylation was performed with 0.4  $\mu$ g PT,  $3 \times 10^6$  cpm  $[^{32}P]NAD^+$ , 10  $\mu$ M  $NAD^+$  (carried over from the first treatment step), 2.5 mM ATP, 1  $\mu$ M GTP, 1 mM EDTA, and 12.5 mM Tris-HCl, pH 7.5, as described in *Materials and Methods*. The reactions were stopped, and incorporation of  $[^{32}P]$ ADP-ribose into  $G_i$  was determined by autoradiography. The figure shows the results of the  $[^{32}P]$ ADP-ribosylation (photographs of the autoradiograms) and the GTPase activity (means of triplicates) of each treatment group. Alb, Nonspecifically labeled BSA (73);  $\alpha_i$ , migration of  $\alpha$ -subunits of  $G_i$ -2 and -3; PTX  $S_1$ , subunit of PT. Note that PT has no effect on the endogenous GTPase activity of  $G_i$ .

the vesicles. Thus, under the conditions used, approximately 70% of the initial  $G_i$  was incorporated into the vesicles in the absence of rhodopsin, and coreconstitution with rhodopsin did not influence its incorporation, as measured by  $[^{32}P]$ ADP-ribosylation of incorporated  $G_i$  (data not shown). Using  $G_i$  ADP-ribosylated in the presence of low specific activity  $[^{32}P]NAD^+$  to monitor the fate of ADP-ribosylated  $G_i$ , incorporation remained at  $70 \pm 5\%$  in both the absence and presence of rhodopsin (not shown). The GTPase activities of vesicles containing  $G_i$  and ADP-ribosylated  $G_i$  were very similar, indicating that incorporation of  $G_i$  into the vesicles is independent of the PT-catalyzed ADP-ribosylation (see below).

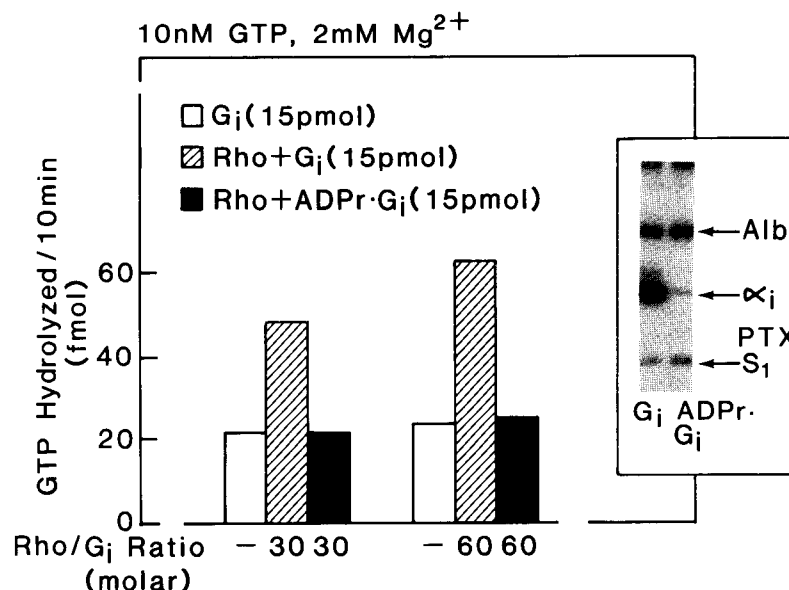


**Fig. 7.** Stimulation of GTPase Activity of  $G_i$  by Rhodopsin upon Coincorporation into Phospholipid Vesicles

A,  $G_i$  (0.5 pmol) was mixed with different amounts of rhodopsin to give the indicated molar protein ratios of 0 (—), 15, 30, and 60 under dim red light. The mixtures were incubated under fluorescent light for 10 min at 32 C in a total volume of 100  $\mu$ l containing 10 nM GTP, 100,000 cpm  $[\gamma\text{-}^{32}P]GTP$ , 3 mM  $MgCl_2$ , 1 mM EDTA, 0.1% BSA, and 10 mM Tris-HCl, pH 7.5, and the  $^{32}P$  liberated was assayed as described previously (46). B, Under dim red light, 15 pmol  $G_i$  alone ( $\square$ ) or  $G_i$  plus increasing concentrations of rhodopsin ( $\blacksquare$ ) were incorporated into phospholipid vesicles, and vesicles were isolated and resuspended as described in *Materials and Methods*. Aliquots (50  $\mu$ l) of vesicles were then incubated under normal laboratory fluorescent light for GTPase activity at 32 C for 10 min in a total volume of 100  $\mu$ l under the same conditions as those in A, and  $^{32}P$  release was measured. Note that only when both proteins are reconstituted into phospholipid vesicles does rhodopsin stimulate the GTPase activity of  $G_i$ . Bars represent means of triplicates. *Inset*, Proportionality between Rhodopsin (Rho) input and stimulation of GTPase.

## DISCUSSION

The present studies, while not providing a final answer as to why receptor-mediated activation of  $G_i$  proteins is blocked by PT, are, however, the first ones to reduce several possibilities to a maximum of two. Thus, our results show the following. 1) Neither the lack of effect of receptors nor that of GTP alone is due to interference



**Fig. 8.** Lack of Effect of Rhodopsin on PT-Treated G<sub>i</sub>

Phospholipid vesicles were prepared as described in *Materials and Methods* using 15 pmol G<sub>i</sub> (G) or ADP-ribosylated-G<sub>i</sub> (ADPr·G) in the absence (□) or presence (▨ and ■) of rhodopsin (Rho) to give the indicated molar ratios of zero (–), 30, or 60. Aliquots (50 μl) of each group of vesicles were incubated for GTPase activity in a volume of 100 μl for 10 min at 32 C in the presence of 10 nM [ $\gamma$ -<sup>32</sup>P]GTP (100,000 cpm/assay), 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1% BSA, and 10 mM Tris-HCl, pH 7.5, and the <sup>32</sup>P, liberated was assayed (46). Results are the means of triplicates. Note that GTPase activity of vesicles that contained ADP-ribosylated G<sub>i</sub> plus rhodopsin is the same as that of vesicles that contained G<sub>i</sub> or ADP-ribosylated G<sub>i</sub> alone. See Fig. 6 for definitions of abbreviations.

with GTP binding to ADP-ribosylated G<sub>i</sub>, as would be the case if PT markedly blocked binding of GTP. The 3- to 5-fold decrease in affinity for GMP-P(NH)P and GTP observed in PT-treated membranes cannot explain the selective lack of effectiveness of GTP, which was not surmountable by high concentrations of GTP. This ruled out an effect of PT on step 2 of the receptor-independent cycle shown in Fig. 2A. 2) The lack of the effect of GTP (either in the absence or presence of agonist) on the activation state of the ADP-ribosylated G<sub>i</sub> is not due to an increase in the intrinsic GTPase activity (*i.e.* the opposite from the effect of cholera toxin-mediated ADP-ribosylation), as seen by direct measurement of this parameter. This ruled out step 3 of both the receptor-independent and the receptor-stimulated cycles of Fig. 1.

Studies from many laboratories have shown that ADP-ribosylation of G proteins in intact membranes lead to a block of their ability to induce the high affinity binding conformer of receptors (38–42), and Kurose *et al.* (30) showed that ADP-ribosylated brain G<sub>i</sub>/G<sub>o</sub> cannot impart high affinity binding to purified muscarinic receptors incorporated into phospholipid vesicles. It has been well established that GDP as well as GTP induce low affinity binding in receptors (50–54). Thus, the PT effect to block receptor action could have been the result of persistent GDP binding or of interference in the ability of the ADP-ribosylated G protein to interact with the receptors. Since the present studies indicate that GDP dissociation is not affected by PT, we conclude that receptor-G protein interaction is a primary parameter

affected by ADP-ribosylation (step 4 of the receptor-dependent cycle shown in Fig. 1, ▨).

The question as to the mechanism by which PT interferes with the effect of GTP in the absence of receptor stimulation is more complex, however. On the one hand, taking the receptor-independent cycle shown in Fig. 1A at face value, the data indicate that the PT blocks step 4 of this cycle, which is the ability of GTP, once bound, to promote the G to G\* transition (▨). On the other hand, it is not clear whether receptor-independent effects even exist. There is evidence to suggest that to activate a G protein, GTP may be absolutely dependent on the cooperative effect of a receptor and that unliganded receptors have basal activity, in which case the receptor-independent cycle shown in Fig. 1 would not exist, and PT action be explained by a simple steric R-G interference.

Indications that receptor-independent activation of a G protein by GTP may not exist come from two different lines of research. One showed that GTP does not affect the conformation of purified G<sub>i</sub>, while GTP $\gamma$ S does; the other showed that receptors affect G proteins in the absence of agonists. The existence of a G to G\* step as a discrete reaction independent of the subsequent subunit dissociation reaction was shown in studies that demonstrated Mg-dependent appearance of tight binding of [<sup>35</sup>S]GTP $\gamma$ S to the trimeric form of the same G<sub>i</sub> preparation as that used in the present study (55). Tight binding of GTP $\gamma$ S was defined as such because the nucleotide remained associated to the protein upon centrifugation through a sucrose density gradient. Tight

binding was associated with a change in the hydrodynamic properties of the protein, which changed its *S* value from about 4 to 3, and a change in its detergent binding, as observed on comparing the sedimentation rates in sucrose density gradients made in H<sub>2</sub>O and D<sub>2</sub>O (55). In contrast, GTP was unable to induce this conformational change. That this lack of accumulation of G<sup>\*</sup>-GTP was not because of return G<sup>\*</sup>-GTP to G-GDP was demonstrated in experiments in which the centrifugation analysis was carried out within 60 min (vertical tube rotor) and the measured rate at which the G<sub>i</sub> preparation hydrolyzed GTP under the conditions used transition was only 0.01 cycles/min (46). Based on this we concluded that with GTP the G to G<sup>\*</sup> transition does not occur. The existence of an agonist-independent activity of receptors emerged from studies in which the effect of incorporating purified  $\beta$ -adrenergic receptor (48) or  $\alpha_2$ -adrenergic receptor (49) into vesicles with G<sub>s</sub> and/or G<sub>i</sub> were studied. In these studies we found that the GTPase activity as well as the rate of GTP $\gamma$ S binding of the incorporated G proteins were lower in the absence than in the presence of receptor. These receptor-enhanced interactions of G proteins with GTP and GTP $\gamma$ S were then stimulated by agonist addition, and the agonist-stimulated GTP hydrolysis or GTP $\gamma$ S binding was reduced to the receptor-enhanced level by specific adrenergic blockers, propranolol in the case of  $\beta$ -adrenergic stimulation by isoproterenol (48), and yohimbine and phentolamine, in the case of  $\alpha_2$ -adrenergic stimulation by epinephrine (49). This, even though it is impossible at this time to determine whether the inability of GTP to activate a purified G protein in solution also applies after the G protein is incorporated into a phospholipid bilayer, it is tempting to suggest that it does and that basal activities of G protein-dependent phenomena are, in fact, not receptor-independent activities but agonist-independent effects of the particular receptors that reside in the membrane under study. In this case, and as mentioned above, the regulatory cycle depicted in Fig. 1A would not exist, and the present studies would have narrowed down the mechanism of PT action to a simple steric hindrance that prevents receptor from interacting with G<sub>i</sub>. Presumably, what was found here for a mixture of G<sub>i</sub>-2 and G<sub>i</sub>-3 also applies to G<sub>i</sub>-1 and G<sub>o</sub>, the prevalent PT substrates in brain (12, 14).

## MATERIALS AND METHODS

### Materials

[ $\alpha$ -<sup>32</sup>P]ATP and [ $\gamma$ -<sup>32</sup>P]GTP were synthesized according to the procedure of Walseth and Johnson (56), and [<sup>32</sup>P]NAD<sup>+</sup> was synthesized by modifications of the methods of Cassel and Pfeuffer (57); they were supplied by the Baylor College of Medicine Diabetes and Endocrinology Research Center. The *n*-octylglucoside and crude soybean phospholipid extract (12% phosphatidylcholine) were obtained from Sigma Chemical Co., (St. Louis, MO) and 95% phosphatidylcholine from soybean was from Avanti Polar Lipids, Inc (Birmingham, AL). Forskolin was obtained from Calbiochem (La Jolla, CA); 3-[3-cholami-

dopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), Lubrol, ATP, and BSA were from Sigma Chemical Co. GTP and analogs, GMP-P(NH)P and GTP $\gamma$ S, were purchased from Boehringer-Mannheim (Indianapolis, IN). Sephadex G-50 (superfine) was obtained from Pharmacia (Piscataway, NJ). The sources of other materials have been reported previously and were of the highest quality available (7, 46, 55).

### Cyc<sup>-</sup> S49 Mouse Lymphoma Cells Membranes

Cyc<sup>-</sup> cells were grown according to the procedures of Bourne *et al.* (58). Membranes were prepared as described by Ross *et al.* (59), except that the membrane preparation was stopped after preparation of the 43,000  $\times$  *g* pellet, and Mg<sup>2+</sup>-free buffers were used throughout.

### Purification of G<sub>i</sub>

G<sub>i</sub> was purified from human erythrocyte membranes as described previously (7, 60) and was approximately 90% pure. Purified G<sub>i</sub> was stored at -70 C in 20 mM mercaptoethanol, 10% Lubrol-PX, 30% ethylene glycol, 1 mM EDTA, 100 mM NaCl, and 10 mM HEPES-Na, pH 8.0. Analysis of the G<sub>i</sub> preparations of G<sub>i</sub> used in these studies using urea gradient/SDS-PAGE followed by Coomassie blue staining and densitometric scanning (61, 62) indicated that they contained approximately 70% G<sub>i</sub>-3 and 30% G<sub>i</sub>-2.

### Purification of Rhodopsin

Rhodopsin was purified from outer segment membranes of bovine retinal rods. Membranes were isolated by the procedure described by Papermaster and Dryer (63) and subjected to sequential washes to remove other membrane proteins, as described by Yamanaka *et al.* (64). Rhodopsin was solubilized in 20 mM CHAPS-containing buffer and purified by Concanavalin-A affinity chromatography following the procedure of Litman (65). All operations were carried out in the cold under dim red light. Spectroscopic analysis before use in reconstitution experiments gave 280/500 ratios between 2.5 and 3.0, indicating that better than 60% of the purified protein was in a form susceptible to activation by light.

### Adenylyl Cyclase Activity

Unless otherwise indicated, incubations were carried out at 32 C for 10 min in a final volume of 50  $\mu$ l containing 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (10–15 cpm), 2 mM MgCl<sub>2</sub>, 100  $\mu$ M forskolin, 0.1% BSA, a nucleoside triphosphate-regenerating system composed of 20 mM creatine phosphate, U/ml, creatine phosphokinase, 0.02 mg/ml (25 U/ml) myokinase, 25  $\mu$ M Tris-HCl (pH 7.6), 5–20  $\mu$ g cyc<sup>-</sup> membrane protein, and the indicated concentrations of GTP, GMP-P(NH)P, or GDP $\beta$ S. The reactions were stopped, and the [<sup>32</sup>P]cAMP formed was assayed by a modification (66) of the method of Salomon *et al.* (67).

### GTPase Assay

Incubations were carried out at 32 C for 10 min in a final volume of 100  $\mu$ l containing 0.5–2 pmol G<sub>i</sub> (50–200 ng protein) or 50  $\mu$ l G<sub>i</sub>-containing vesicles (~100 ng protein, assuming 70% incorporation of G<sub>i</sub> into phospholipid vesicles), 100,000 cpm [ $\gamma$ -<sup>32</sup>P]GTP, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1% BSA, 10 mM ethanolamine, 10–100 nM GTP, and 10 mM HEPES-Na, pH 8.0. The reactions were stopped, and the <sup>32</sup>P<sub>i</sub> released was measured as described previously (46).

### Activation of PT

PT (68) was dialyzed (11) and activated for 30 min at 32 C in a final volume of 30  $\mu$ l containing 50 mM dithiothreitol and 0.025% BSA at a concentration of 200  $\mu$ g/ml.

# PT Treatment of Cyc<sup>-</sup> Cells and Preparations of Control and PT-Treated Membranes

Cells were treated or not with 0.1  $\mu$ g/ml unactivated PT for 15 h, and membranes were prepared as described in Hildebrandt *et al.* (11). Membranes from control and treated cells were stored at  $-70^{\circ}\text{C}$  until used (3–4 months) without loss of inhibitory effects of GMP-P(NH)P. The effectiveness of the treatment of intact cells with PT was evaluated by ADP-ribosylation of the isolated membranes (10  $\mu$ g protein) with activated PT (see below). Labeling intensities obtained with membranes from PT-treated cells (Fig. 2) were less than 5% of those obtained with membranes from control cells.

## ADP-Ribosylation of Membrane G<sub>i</sub>

Ten microliters of activated PT (6  $\mu$ g) were incubated with 20  $\mu$ l membranes (10  $\mu$ g protein) from control or PT-treated S49 cells, 1 mM EDTA, 0.1% Lubrol PX, 20 mM Na-HEPES (pH 8.0), and 30  $\mu$ l medium containing 20  $\mu$ M [<sup>32</sup>P]NAD<sup>+</sup> ( $4\text{--}6 \times 10^6$  cpm), 0.2 mM ATP, 2 mM GTP, 2 mM EDTA, and 25 mM Tris-HCl (pH 7.5) for 45 min at 32  $^{\circ}\text{C}$ . The reaction was stopped by the addition of 40  $\mu$ l 375 mM NaCl containing 10  $\mu$ g BSA, followed by precipitation with 900  $\mu$ l ice-cold acetone, centrifugation, and extraction of precipitated [<sup>32</sup>P]NAD<sup>+</sup> with 10% trichloroacetic acid (TCA). After removing TCA from the precipitates, protein pellets were dissolved in Laemmli's sample buffer and subjected to 10% SDS-PAGE. The gels were stained with Coomassie blue to insure equal sample recovery as indicated by the BSA band, destained, and autoradiographed. For further details see the reports of Ribeiro-Neto *et al.* (69) and Scherer *et al.* (62). During these procedures, some of the [<sup>32</sup>P]NAD<sup>+</sup> becomes incorporated into BSA, possibly by photoactivation of the nucleotide, giving rise to a nonspecifically labeled band at 67K.

## ADP Ribosylation of Purified G<sub>i</sub>

Ten microliters of activated PT (6  $\mu$ g) were incubated with 20  $\mu$ l G<sub>i</sub> (3  $\mu$ g protein) in a final volume of 60  $\mu$ l containing, unless otherwise indicated, 2 mM NAD<sup>+</sup>, 0.1 mM ATP, 1  $\mu$ M GTP, 1 mM EDTA, and 12.5 mM Tris-HCl, pH 7.5, for 45 min at 32  $^{\circ}\text{C}$ . As controls, PT was incubated under the same conditions without G<sub>i</sub>, and G<sub>i</sub> was incubated under the same conditions with PT activation buffer without the toxin. To test for effectiveness of the ADP-ribosylation of G<sub>i</sub>, an aliquot of ADP-ribosylated G<sub>i</sub> (2–10  $\mu$ l) was diluted 20 times with 1 mM EDTA and 10 mM Tris-HCl, pH 7.5, and subjected to a second ADP-ribosylation in the presence of [<sup>32</sup>P]NAD<sup>+</sup>. The second incubation was in a final volume of 100  $\mu$ l containing 10  $\mu$ l of the diluted sample, 20  $\mu$ l 20  $\mu$ g/ml activated PT, 10  $\mu$ l [<sup>32</sup>P]NAD<sup>+</sup> ( $3\text{--}5 \times 10^6$  cpm; 200–400 Ci/mmol), 2.5 mM ATP, 1  $\mu$ M GTP, 1 mM EDTA, and 12.5 mM Tris-HCl, pH 7.5, for 45 min at 32  $^{\circ}\text{C}$ . The reaction was stopped and processed as indicated above for ADP-ribosylation for membranes, and incorporation of [<sup>32</sup>P]ADP-ribose into G<sub>i</sub> was determined by autoradiography after separation of G<sub>i</sub> by SDS-PAGE.

## Preparation of Phospholipid Vesicles

A modification of the procedure described by Ross and collaborators (70, 71) was used. Phospholipids were dissolved in chloroform-methanol (2:1) at a concentration of 17 mg/ml. One hundred and fifty microliters of the crude soybean phosphatidylcholine extract and 50  $\mu$ l phosphatidyl choline from Avanti Polar Lipids were mixed in a 15-ml Corex tube and dried under nitrogen. The phospholipids were then resuspended in 65  $\mu$ l 1 M octylglucoside, and 200  $\mu$ l buffer A (100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 20 mM  $\beta$ -mercaptoethanol, and 10 mM Na-HEPES, pH 8.0), vortexed, and incubated for 10 min at 4  $^{\circ}\text{C}$  until completely dissolved. All subsequent operations before incubation for GTPase activity were carried out in a cold room

under dim red light. The following were added to the phospholipid/octylglucoside suspension: 300  $\mu$ l of a mixture of 20  $\mu$ l 10% BSA, 30  $\mu$ l control G<sub>i</sub> or ADP-ribosylated G<sub>i</sub> (15 pmol), 30–120  $\mu$ l rhodopsin when indicated, and 130–220  $\mu$ l buffer A (giving final concentrations of approximately 11.3 mM phosphatidyl choline, 115 mM octylglucoside, and 0.18% Lubrol-PX). The reconstitution mixture was applied to a 33-ml Sephadex G-50 column (id, 0.9 cm) and chromatographed with buffer A at a flow rate of 0.2–0.3 ml/min. The void volume (2–3 ml) was collected, and vesicles were concentrated and separated from unincorporated protein by ultracentrifugation in a Ti75 Beckman rotor for 2 h at 45,000 rpm (180,000  $\times g$ ). The pellets were resuspended with 500  $\mu$ l buffer A and assayed under normal fluorescent laboratory light.

The data presented here are representative results of experiments that were repeated at least three times to insure reproducibility.

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